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APPENDIX D

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/163,272	09/29/1998	JONATHAN DINSMORE	DNI-041CPA	9801

7590 07/16/2002

AMY E. MANDRAGOURAS
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BOSTON, MA 02109

EXAMINER
BAKER, ANNE MARIE

ART UNIT	PAPER NUMBER
1632	25

DATE MAILED: 07/16/2002

OIPE JAN 21 2003 PATENT & TRADEMARK OFFICE

Oct 16, 2002 RESPONSE DUE
Jan 16, 2003 ESP

Please find below and/or attached an Office communication concerning this application or proceeding.

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JUL 19 2002
RETRIEVED: 7/22 Mac
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PTO-90C (Rev. 07-01)

Office Action Summary	Application No.	Applicant(s)	
	09/163,272	DINSMORE, JONATHAN	
	Examiner	Art Unit	
	Anne Baker	1632	

-- Th MAILING DATE of this communication app ars on the cover she t with the correspondenc address --

Peri d for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *03 May 2002* .

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1,3-8,10-18,20-26 and 28-47* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1,3-8,10-18,20-26 and 28-47* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _____ .
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☒ Other: *detailed action* .

DETAILED ACTION

The amendments filed February 8, 2002 (Paper No. 17) and May 3, 2002 (Paper No. 22) have been entered. Claims 1, 3, 4, 10, 18, 20, 21, and 36 have been amended. Claims 2 and 19 have been cancelled. Claim 48 has been newly added.

Claims 1, 3-8, 10-18, 20-26, and 28-48 remain pending in the instant application.

The following rejections are reiterated or newly applied and constitute the complete set of rejections being applied to the instant application. Rejections and objections not reiterated from the previous Office Action are hereby withdrawn.

Continued Prosecution Application

The request filed on May 3, 2002 (Paper No. 20) for a Continued Prosecution Application (CPA) under 37 CFR 1.53(d) based on parent Application No. 09/163,272 is acceptable and a CPA has been established. An action on the CPA follows.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 18, 20-26, 28-37, 43, 44, and 46 stand rejected and Claims 1, 3-8, 10-17, 38-42, 45, 47, and 48 are rejected under 35 U.S.C. 112, first paragraph, for reasons of record advanced on pages 3-9 of the Office Action of Paper No. 6 (mailed 12/7/99), on pages 2-3 of the Office Action of Paper No. 11 (mailed 10/18/00), and on pages 2-3 of the Office Action of Paper No. 14 (mailed 7/3/01), because the specification, while being enabling for a method of treating a xenogeneic subject having spinal cord

damage arising from amyotrophic lateral sclerosis, does not reasonably provide enablement for treating a xenogeneic subject having spinal cord damage arising from the claim-designated neurodegenerative disorders, spinal cord injuries, or aging. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims.

Claims 1, 3-8, 10-17, 38-42, 45, 47, and 48 are directed to compositions. However, the claims recite an intended use. As such the specification must provide an enabling disclosure for the intended use. Moreover, the intended use must be enabled for its full scope.

The specification fails to provide an enabling disclosure for the method of cell-based therapy because methods of transplantation of neural tissue are not routinely successful and the specification does not offer adequate guidance to enable one skilled in the art to practice the claimed invention over the full scope to derive a therapeutic benefit in a diseased animal. The specification teaches that the only use for the claimed compositions and the claimed method of transplantation is to produce a therapeutic effect, but the specification does not adequately teach how to use the claimed compositions for their intended use, over the full scope, nor how to use the claimed method to produce such an effect. Jackowski et al. (1995) details the limitations and unpredictability associated with the transplantation of neural tissue. At page 311, column 1, paragraph 2, the reference discusses barriers to successful transplantation of neural tissue, notably the presence of molecules that actively inhibit the regeneration of mammalian CNS and PNS axons. The specification does not offer adequate guidance as to how the claimed method could be used therapeutically over the full scope for the treatment of the wide variety of disorders recited in the claims. The working examples are limited to producing a therapeutic effect in an ALS model. Other than this, the specification provides general teachings only, but does not provide specific guidance for treating other pathological conditions. The specification fails to provide guidance relating to the number of cells to

inject, the site of injection, and the extent of cellular persistence required and attainable in practice, to provide a therapeutic benefit for the treatment of any other pathological disorder.

Given the lack of specific guidance in the specification directed to the wide variety of disorders recited in the claims, the broad scope of the claims, and the limited working examples directed to producing a therapeutic effect upon transplantation of porcine spinal cord cells into an animal model of ALS, one of skill in the art would have been required to engage in undue experimentation to practice the claimed method over the full scope and use the claimed compositions for their intended use, over the full scope.

Conclusion

No claims are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Anne-Marie Baker, Ph.D. whose telephone number is (703) 306-9155. The examiner can normally be reached Monday through Thursday and alternate Fridays from 10:00 AM to 7:30 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Deborah Reynolds, can be reached on (703) 305-4051. The fax phone number for the organization where this application or proceeding is assigned is (703) 308-8724.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the patent analyst, Dianiece Jacobs, whose telephone number is (703) 305-3388.

Anne-Marie Baker, Ph.D.

Anne-Marie Baker
ANNE-MARIE BAKER
PATENT EXAMINER

Notice of References Cited	Application/Control No. 09/163,272	Applicant(s)/Patent Under Reexamination DINSMORE, JONATHAN	
	Examiner Anne Baker	Art Unit 1632	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
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	J	US-			
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	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	jackowski Neural injury repair: hope for the future as barriers to effective CNS regeneration become clearer 1995 pp.303-317
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

British Journal of Neurosurgery (1995) 9, 303–317

REVIEW ARTICLE

Neural injury repair: hope for the future as barriers to effective CNS regeneration become clearer

ANDRE JACKOWSKI

Department of Neurosurgery, University of Birmingham, Birmingham, UK

Abstract

In this review the author outlines the early history of clinical and scientific research upon the inability of the CNS in man to successfully regenerate following injury. As we proceed into the 21st Century we have gained a far greater understanding of the molecular biology, pathology and other factors that lead to the adult CNS being non-supportive and indeed actively inhibitory to axonal regrowth. On the basis of these recent advances in knowledge, the author outlines possible therapeutic approaches that may enable more effective CNS regeneration to be accomplished in the future.

Key words: *Axonal regeneration, CNS injury, neural repair, neurotrophic factors.*

> On various occasions, basing ourselves on precise observations by ourselves and others, we have recorded the radical incapacity of central axons, medullated or non-medullated, young or old, to restore interrupted paths of the white and grey matter. In the spinal cord, under propitious circumstances, one sees from time to time cones of growth connected with axons of the white matter and capable of ramifying and growing across the scar. But in the cerebellum and cerebrum this vigorous, though ineffective, attempt to innervate the cicatricial connective tissue is always lacking.
>
> In the nerves the restoration is a revolutionary work, begun with the utmost rapidity and activity, and apparently stimulated by obstacles. In the centres, on the contrary, the apathy or precarious productive attempts of the first few days are succeeded by the most absolute inactivity.
>
> Ramon y Cajal, 1914[1]

Introduction

That certain injuries of the central nervous system in man failed to recover and could not be effectively treated was recognized as far back as 4500 years ago by the physicians of ancient Egypt,[2,3] and by Hippocrates some 2000 years later.[4] During the late 19th century, the evident lack of any significant regenerative repair within the CNS of man and other adult mammalian species attracted considerable attention from the many eminent neuroscientists of this period, that included Brown-Sequard, Stroebe, Bielschowsky, Marinesco and Ramon y Cajal.[1,5–8] This period of intensive observation culminated

0268-8697/95/030303–15

with the publication of Cajal's classic treatise on the subject "Degeneration and regeneration of the nervous system".[1] Cajal confirmed that the severed ends of CNS axons initially attempt to regenerate with the formation of growth cones similar to those observed in divided peripheral nerves, but that this early outgrowth was not maintained. A scientific giant, he was considerably ahead of his time in that he correctly predicted the existence both of neurohumoral growth factors and the more permissive nature of the Schwann cell environment as being of vital importance in the differing regenerative capacities of the CNS and peripheral nervous system (PNS).

Peripheral nervous system vs central nervous system

Unlike CNS injuries, damage to the adult mammalian peripheral nervous system, provided approximation of cut neural ends is maintained, usually results in effective regrowth of axonal processes and some degree of useful recovery. Distal to injury of a PNS nerve, a succession of changes leading to neural repair takes place as originally described by Waller in 1852, cited by Cajal.[1] The processes that take place during anterograde Wallerian degeneration have subsequently been investigated and described in great detail by a number of investigators.[9–16] Essentially, axons together with their myelin sheaths degenerate, myelin debris being removed by macrophages and Schwann cells, leaving behind largely intact endoneurial tubes that consist of a basal lamina and connective tissue. During the period in which myelin breakdown and removal occurs, Schwann cells proliferate within the endoneurial sheaths forming longitudinally continuous columns comprised of Schwann cells together with their overlapping elongated processes. The proximal axons undergo regenerative sprouting, usually with four to eight new processes emerging from the proximal stump.[9] The growth cones that lead the regenerating axons, grow towards and into the Schwann cell columns which seem to act both as axonal attractors and directional guides for the regenerative repair process.

Comparative anatomy

Unlike the situation that exists in man and other mammalian species, many adult submammalian vertebrates receiving a CNS injury, such as a complete transection of the spinal cord, can undergo successful regeneration. Axons grow across the injury site and beyond to achieve some degree of functional recovery. Spinal cord regenerative repair has been documented in fish, amphibians and some reptiles by numerous investigators from the latter half of the nineteenth century onwards. For a fully comprehensive review of this early work see Clemente.[17] Within such submammalian species there appears to be a far greater degree of cellular plasticity within the CNS. The neural regeneration that is achieved following spinal cord transection appears to be mediated by an interaction between ependymal cells and the exposed overlying mesenchymal cells.[18–21] In the early weeks after spinal injury, ependymal cells around the central canal redistribute and proliferate. These, together with mesenchymal cells derived from connective tissues around the lesion site, bridge the gap between the cord stumps. CNS axonal growth cones appear readily able to cross such tissue, the ependymal tube serving to guide the regenerating axons.[20]

Embryogenesis

During embryonic development, growing CNS nerve fibres successfully negotiate many intervening structures, often travelling considerable distances to reach their target tissues. Whilst to some extent axonal outgrowth may reflect a predetermined 'growth axis' possessed by the neuron itself,[22] it may aid our understanding of abortive CNS regeneration to consider, first, some of the extrinsic factors that are believed to be important in guiding and encouraging neural growth at this earlier time.

Electrical fields

Ariens Kappers[23] developed Strasser's 1892 electrical theory[24] to formulate his concept of neurobiotaxis. A similar electrodynamic theory of neural development was elaborated by Burr.[25] These theories held in common the proposition that dendritic and axonal growth is influenced by electrical fields. Activation of one part of the nervous system, stimulated by another, very clearly plays an important part both in modifying neuronal development and indeed in neuronal survival.[26,27] There is, however, little convincing experimental work to support the hypothesis that differences in electrical potential *per se* have any significant effect upon either the direction of growth or orientation of CNS axons.

Chemotropism

Chemotropic attraction of the growing tips of axons by diffusible factors secreted from target areas was first proposed by Ramon y Cajal in 1892.[28] This concept was elaborated and later refined, forming the basis of the chemoaffinity hypothesis of Sperry, in which growing axons are believed to recognise their topographical targets on the basis of positional markers in the form of gradients of molecules distributed along primary axes.[29] It would be immensely complex and it appears unlikely that chemotropic pathways alone could guide the growth cone of a growing axon all the way from say a parent neuron in the motor cortex to a spinal motorneuron in the lower dorsal spinal cord (see discussion by Novak and Bolz).[30] There seems, however, little doubt that chemotropic attraction, either by released diffusible or by membrane-bound molecules in target and guide tissues, plays an important role both in the final target approach for growing axons in the developing CNS and again later in life for regenerating axons in the PNS.

Mechanical substratum/adhesive molecules

There is considerable evidence that guidance by mechanical and by contact factors can influence the growth and orientation of axonal processes. Harrison[31] and Ramon y Cajal[32] were two early workers who stressed the importance of pre-existing mechanical structures in directing axonal growth. Rakic's studies[33,34] have shown that axons growing in the developing cerebellum, associate preferentially with radially arranged glia. Similarly, neuroblast migration in the early development of the CNS appears to be mechanically guided by an alignment of glial processes. In the developing CNS the extracellular spaces are greater, and they form orientated intercellular channels that may more readily permit axons to pick a way through towards a specified target, than would be the case in the mature CNS.[35,36] Weiss[37,38] demonstrated that axons growing in tissue culture became preferentially orientated, parallel to the direction of tension forces set up within the substrate growth medium. Weiss called this phenomenon contact guidance. Axonal growth, both during development and during regeneration, is accomplished by growth cones located at the tips of elongating axons.[31,39,40] These are motile pathfinding structures and the region at which new membrane is added to accomplish axonal elongation.[41] Adhesive interactions between the growth cone of a growing axon and the substratum within which it is growing appear to be critically important both for directional growth and the extent of axonal elongation, contact guidance and contact inhibition occurring.[42–45] See also further discussion under substrate adhesion/neurite-promoting factors.

Theories on the causes of regeneration failure in mammalian CNS

These can be considered under the broad headings of:

1. Intrinsic inability of CNS neurons to mount a regenerative response.
2. A CNS environment that is non-supportive or actively inhibitory to neural regeneration.

Intrinsic inability of central neurons to mount a regenerative response

Somal reaction. Neurons wholly contained within the CNS are referred to as central neurons, whilst the term peripheral neuron is used to include all neurons whose processes lie wholly or partly in a peripheral nerve. Following transection of the axon of a vertebrate peripheral neuron, its cell body undergoes a series of changes referred to as the retrograde neuronal response. Initially thought to represent a degenerative response, it is now known that it enables the regenerative response to be mounted. Granular endoplasmic reticulum is broken-down and together with free ribosomes is redistributed towards the cell periphery. Nuclear RNA synthesis is increased together with an increase in cytoplasmic protein synthesis.[46,47] Reactive glial changes occur around the axotomized peripheral neurons with proliferation of microglial cells and hypertrophy of astrocytes. How the cell body receives the message that axonal continuity has been interrupted remains uncertain, but it is presumed that the signal is carried back to the soma by retrograde axoplasmic transport.[48,49] Central neurons react to axotomy in a less consistent manner. Initially, some do exhibit a series of cytological changes very like those seen in peripheral neurons. Subsequently, however, they show a progressive decline in levels of both cytoplasmic and nucleolar RNA content.[50,51] Periods of axon outgrowth during normal neural development and also the time of regrowth in axonal regeneration are characterised by the selective expression of certain specific genes by neurons. These genes encode for proteins which are produced in high levels and transported down into growth cones that are responsible for neurite extension.[52] Growth-associated protein GAP-43 is one such protein that accumulates in neuronal growth cones and is presumed to be an essential building-block for axonal elongation.[53,54] Submammalian CNS or PNS injury and also mammalian PNS injury result in a greatly elevated GAP-43 expression, roughly coincident with the initiation of axon regrowth.[52] In contrast, very little elevation in GAP-43 levels takes place after axotomy of adult mammalian CNS neurons.[55] Although an increase in cellular content of RNA and GAP-43 induction following axotomy is the hallmark of those neurons capable of axon regeneration, while the converse holds for non-regenerating neurons, it is unclear whether this is a cause or the result of ineffective neural regeneration.

Evidence of an initial abortive regeneration by many central neurons. The earliest investigators, in contrast to their findings in submammalian species, reported no signs of regeneration following an injury to the mammalian CNS.[17] According to Clemente,[17] Kahler in 1884 concluded that this was because the CNS contained no Schwann cells. The earliest description of the abortive mammalian CNS regenerative response belongs to Stroebe in 1894.[6] He observed that some regenerating nerve fibres did cross the scar tissue of the transected spinal cord but that they failed to achieve any true restitution of spinal cord tissue. Bielschowsky,[7] Cajal[9,56] and others confirmed these findings. Cajal in particular with his superb histological techniques was able to clearly demonstrate after spinal cord section in mammals, that the proximal stumps of large numbers of transected axons sprouted new axonal processes possessing typical terminal growth cones. After approximately a month, however, these regenerating axons atrophied and ultimately degenerated.[9,56] Cajal came to the conclusion that this was not due to an intrinsic inability of CNS neurons to regenerate or the presence of a neuroglial scar, but rather the absence of a trophic and orientating environment similar to that produced in the lesioned PNS by the proliferated cells of Schwann. Similar findings have been found by more recent investigators[57] with the concept now firmly established of an early regenerative effort by the CNS, but that generally the regenerating axons of central neurons appear unable to continue growing across the transection site and beyond.[17]

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Ability of certain mammalian central neurons to regenerate successfully. Within the CNS, certain adrenergic, noradrenergic, dopaminergic and serotonergic neurons seem to be capable of sustaining a more prolonged regenerative growth response.[58–60] After physical or chemical axotomy their axons undergo regenerative sprouting from the cut ends, with the newly formed axons regrowing within the CNS often for considerable distances. Perhaps related to this ability is a similarly seen ability of monoaminergic axons to undergo a more marked degree of collateral sprouting than is seen with other central neurons.

Peripheral nerve 'bridge' experiments. Tello performed one of the earliest recorded (1911) PNS to CNS implantation experiments.[61] A co-worker of Cajal's, his investigation was prompted by a theory of Cajal that a neurohumoral agent, released by Schwann cells, was partly responsible for the successful regeneration seen in the PNS. Tello implanted predegenerated grafts of sciatic nerve into the mammalian cerebrum and after approximately 2 weeks, noted extensive growth of 'central' fibres into the graft, cited by Clemente.[17] Subsequent workers obtained similar findings using peripheral nerve segments grafted into the CNS either in spinal cord, cerebral or optic nerve injury models.[62–64] During the 1970s, Kao[65–67] performed an elegant series of experiments after first developing a delayed microsurgical grafting technique to reduce scarring and necrosis at the PNS graft/spinal CNS interface. Using this technique, sciatic nerve segments inserted between the ends of a transected spinal cord were invaded by regenerating axons that readily bridged the gap between the cord stumps. Myelination of regenerated axons was also observed. Although this and previous authors' studies all showed significant invasion of CNS-implanted peripheral nerve grafts by regenerating axons, it was not possible to demonstrate firmly if these were derived from central neurons, peripheral neurons or autonomic nerve fibres. More recently, however, the application of retrograde neuroanatomical tracing methods by Aguayo and his co-workers has now firmly established that central neurons in the spinal cord, cerebrum, medulla and retina of adult mammals can extend axons for distances equal to the longest CNS fibre pathways in these animals, through such peripheral nerve graft 'bridges'.[68–71] These findings have now clearly refuted the notion that central neurons are intrinsically incapable of mounting any effective regenerative responses after injury.

CNS environment non-supportive/inhibitory to neural regeneration

Evidence for the hypothesis.

Ability of CNS axons to penetrate PNS grafts but not readily be able to re-enter the CNS. As outlined above, the results obtained from the more recent PNS to CNS implantation experiments conclusively demonstrate that the cut axons of central neurons are eminently capable of regenerating over considerable distances when routed away from the CNS along peripheral nerve grafts. When, however, these same regenerating central axons reach the end of the PNS graft and contact the distal CNS-graft junction, they generally either fail to traverse the PNS–CNS interface or if they do successfully re-enter the CNS they at best grow only 1–2 mm.[70,72]

Inability of regenerating PNS axons to penetrate into CNS. Evidence of the generally non-permissive nature of the CNS environment towards axonal regeneration is also provided by studies that involve the dorsal sensory root and its attachment to the spinal cord. Projecting into the dorsal root for some 100–1000 μm is a conical transition zone (TZ) consisting of interlacing islands of central and peripheral nervous tissue.[73,74] When the central branch of a dorsal root is interrupted by crushing, or severance with immediate reanastomosis of its cut ends, the divided axons successfully regenerate through the lesion their growth unimpeded within the columns of reactive Schwann cells and their basal laminae. When, however, these vigorously growing axons encounter the

CNS at the TZ, the majority either stop completely or turn back towards the periphery.[74–77] Similar findings are obtained if a ventral motor root is divided and coapted to the central process of a divided dorsal root. The motor axons regenerate along the dorsal root, but again cease to progress when they encounter CNS tissue at the TZ.[78]

In vitro *experiments. In vitro* studies, in which dissociated peripheral sensory or sympathetic neurons are confronted with explants either of adult rat PNS (sciatic) or CNS (optic) nerves show that these differences in regenerative growth capacity within peripheral or central nervous tissue environments, persist also in tissue culture.[79] In the same cultures, in which up to several hundred axons could be found in the sciatic nerve explants, neurite ingrowth into optic nerves was completely absent.[80] Cryostat-cut sections of the spinal cord, used as an *in vitro* substrate, similarly support only minimal neurite outgrowth as compared with tissues taken from the peripheral nervous system.[81]

Foetal transplants seem exempt. A clear exception to the non-permissive nature of the adult CNS towards axonal growth, is the target-specific and long-distance fibre outgrowth achieved by some transplanted foetal neurons. Studies by Bjorklund and co-workers,[82,83] Tonder,[84] Wictorin,[85] Stromberg[86] and Raisman's group[87] have shown that donor embryonic hippocampal, or septal neurons from more than one species and also human neuroblasts, when transplanted into an adult mammalian CNS environment can form axons which appear able to grow into and innervate either local or distal target fields within a host animal (*vide infra*).

The conclusions that we can draw from all of the above is that with the exception of certain monoaminergic neurons, weakly myelinated systems, and foetal transplants, the adult mammalian CNS presents a microenvironment that is non-supportive or, possibly, actively inhibitory to the regenerative growth repair capacity of both peripheral and central neurons. In contrast, a PNS-type environment seems capable of supporting and directing axonal regeneration, not only by peripheral neurons but by central neurons also.

Factors possibly leading to a non-supportive or inhibitory CNS environment

Relative lack of neuronal growth factors. The discovery of a Nerve Growth Factor (NGF) by Levi-Montalcini, Cohen, Hamburger, and colleagues[88] that caused a dramatic increase in the growth of certain neurons, confirmed the far-sighted prediction of Ramon y Cajal half a century earlier. It was followed subsequently, by the discovery of numerous other growth factors. Neuronal growth factors can exert their effects either through an enhancement of neuronal survival, by promoting axonal growth extension by the neuron or in some instances via both mechanisms. During development, excess numbers of neurons are produced that subsequently are reduced to adult numbers (down by 80% in the case of cholinergic spinal cord motor neurons) by a naturally occurring phenomenon of developmental neuronal death.[89] This takes place around the time when axons reach their target areas and led to the concept that the establishment of successful axon-target contact leads to the protection of that neuron from developmental death. The contacted target tissue producing one or more, necessary neurotrophic factors. Sympathetic and most, if not all, neural crest-derived sensory neurons require NGF for survival during embryonic and early postnatal life,[90] NGF synthesis at this time being primarily by the target tissue for that particular neuron. More recent studies have shown that NGF receptors are also widely present and play a significant role in mammalian CNS development, particularly in the case of cholinergic neurons.[90]

A number of other growth factors with neurotrophic activities *in vitro* have now been identified. These include: brain-derived growth factor (BDGF), ciliary neurotrophic factor (CNTF), epidermal growth factor, acidic fibroblast growth factor (FDGF), basic

FDGF, prototypic nerve growth factor, neurotrophin-3 (NT-3), midkine, pleiotrophin and insulin-like growth factors.[90–93]

Investigations upon neuronal cells growing in culture have emphasized the importance of an additional group of substrate-bound factors present in the extracellular matrix or within the membranes of cells, lacking in any direct neurotrophic activity, but which can promote neurite adhesion and extension. Neurite-promoting factors (NPFs) include laminin, fibronectin, N-Cam, GM_1 ganglioside and contactin (for reviews see Lipton,[94] Sonderegger and Rathjen.)[95] The precise role of these large molecular weight NPFs in directing development and in the regeneration of CNS axons remains yet to be determined, but they are clearly ideal candidates for the selective guidance of growing axons towards their target areas.[96]

In maturity, transection of a peripheral nerve results in large quantities of NGF being produced by the supporting Schwann cells that proliferate within the distal nerve stump. This synthesis requires an interaction with macrophages, which invade the nerve to phagocytose degenerate myelin, and is apparently regulated by the macrophage-derived lymphokine, interleukin 1.[97–100] Transection of peripheral nerves has also been found to lead to a massive increase in local BDNF mRNA levels, with both NGF and BDNF being known to stimulate the regeneration of axons from adult mammalian PNS neurons.[101] Schwann cells furthermore, are a rich source of CNTF-like neurotrophic activity.[102] Isolated CNS astrocytes in culture can also express a variety of neurotrophic factors, (NGF, CNTF, BDNT, NT-3 and FDGF) which are capable of acting upon cultured neurons from both the PNS and the CNS.[103] *In vivo*, however, neuronal growth factors within the normal CNS are predominantly localized to neuronal cell populations.[104,105] CNS injury causes a time-dependent increase in neuronotrophic activity at a lesion site.[105] The appearance of neuronotrophic factors in the mammalian brain after an injury occurs at a slower rate in adults than neonates and to a lower final level of biological activity.[105,106] This latter finding may well account for why CNS implants in adult brains survive and function less well than in newborn animals. A key difference between the peripheral and central nervous system, of relevance to the success or otherwise of regeneration, may be a greater ability of Schwann cells to produce neurotrophic factors in large quantities. Certainly, exogenously supplied neurotrophic factors are able to promote central neuron survival and nerve regrowth following *in vivo* injury.[107–110] The massive increase in BDNF mRNA seen after lesioning peripheral nerves is of special interest in view of the demonstrated ability of peripheral nerve grafts and of isolated Schwann cells to enhance CNS regeneration, particularly in the case of retinal ganglion cells which are supported by BDNF, but not by NGF.[102] Soluble components released by lesioned peripheral nerves can both prevent cell death and induce substantial axonal elongation from isolated retinal ganglion cells in a manner very similar to that seen with exogenous BDNF.[111] A deficient availability of trophic factors for adult central neurons is considered generally by a number of authors to be one of the causes for the relative lack of success of CNS axons in achieving regeneration.[112]

Presence of neurite growth inhibitory factors. Mechanical injury to the adult mammalian CNS always results in the formation, at the lesion site of a dense scar, consisting of elements both of a fibroblast-derived collagenous nature and a glial scar composed of reactive astrocytes together with their cytoplasmic processes.[113–115] Reactive astrocytosis also occurs remote from the site of an injury, in response to CNS demyelination such as following Wallerian degeneration, and also in multiple sclerosis and the degenerative diseases. In contrast, lesions of the foetal and early neonatal mammalian CNS appear to provoke little if any scarring.[116–118] Astrogliosis within the injured CNS includes both a proliferative response, hypertrophy, and an increase in the number of cytoplasmic processes. It is charac-

terised by extensive synthesis of glial fibrillary acidic protein (GFAP) a protein subunit of glial intermediate filaments.[119,120] The accumulation of astrocytes at the lesion margins, results in the formation of an astrocytic boundary or external glial limitans—thicker than that found in the normal CNS glia limitans.[116,120,121] The formation of a glial/connective tissue fibrous scar following trauma is clearly beneficial, in that it re-establishes the integrity of the CNS, sealing it off from the external environment and the inherent risk of infection. The glial/fibroblastic scar has, however, long been considered to represent an impenetrable physical barrier to the regenerative response of CNS axons, largely on the finding that abortively regenerating axons are found within such scar tissue.[9,113] More recent evidence would challenge this view and suggests that the CNS scar does not simply represent a mechanical obstruction to the path of regenerating axons,[122] but instead, inhibits regeneration at the molecular level through cell surface contact-mediated interactions. As reviewed earlier, following division of the central branch of a dorsal root, the interrupted sensory axons successfully negotiate the resultant PNS fibroblast-derived connective-tissue scar, but are halted at the CNS environment of the dorsal root transition zone (DRTZ), where there is a complete absence of fibrous scar tissue, but wherein the contained astrocytes respond to degeneration of the dorsal root afferents by undergoing typical astrogliotic reactive changes.[123] Another challenge to the concept of the CNS scar as being a major physical constraint to axonal regeneration has come from the work of Ann Logan and her colleagues. Their work demonstrates that transforming growth factor B1 (TGF-B1) is one of the first growth factors to be expressed at the site of CNS injury[124] and that it is an important orchestrator of scar production. Neutralization of TGF-B1 activity in a CNS wound can completely prevent the formation of fibrous scar material, yet even with such inhibition, damaged adult axons are still incapable of regenerating across a lesion.[125] By what mechanism then, might reactive astrocytes halt the further growth of regenerating axons in the CNS? In the DRTZ, the growth cones of regenerating axons stop and make stable synaptoid terminals among the processes of reactive astrocytes.[74,77,126] Recently, Liuzzi and Lasek[77,127] have proposed that reactive astrocytes halt the growth of regenerating axons in the mammalian spinal cord by activating an intrinsic physiological stop-pathway that is normally activated in developing axons when axonal growth cones make contact with their appropriate target neurons or peripheral receptors. Other mechanisms whereby reactive astrocytic processes might stop axonal outgrowth have been reviewed by Stensaas.[74] More recently, McKeon and colleagues[128] have demonstrated that there is an increased expression by reactive astrocytes, of tenascin and chondroitin sulphate proteoglycan, cell surface molecules that are inhibitory towards axon growth.

It has been commented upon that some unmyelinated adult CNS axons and neonatal CNS axon systems prior to myelination, but not postmyelination, possess a substantial regenerative capacity.[129] Retinal ganglion cell axons in mammals with unmyelinated retinas, when injured near their cell bodies, are capable in the absence of intact or degenerate myelin of growing for several mm; in contrast, within the myelinated optic nerve environment there is a complete lack of regeneration of these same axons.[130,131] Similarly, unmyelinated hypothalamic neurosecretory fibres within the pituitary stalk can regenerate across the lesion if the pituitary stalk is sectioned.[132,133] These same axons, however, will not regenerate if the cut stalk is approximated to other CNS tissues that contain myelin.[134] Reviewing the above and other experimental findings, Berry[129] proposed the hypothesis that following CNS injury, proteolytic breakdown of mammalian CNS myelin releases axonal growth inhibitory factors (AGIFs) and that these are responsible for the abortive growth response of most axons within the CNS. Schwab and co-workers[90,135-139] in a series of studies, identified in both CNS myelin and oligodendrocyte membranes, two minor

proteins in the molecular weight fractions 33–35 and 250 kDa, respectively, with strong inhibitory effects upon growing neurites (neurite growth inhibitors NI-35 and NI-250). The application of a monoclonal antibody (IN-1) raised against these proteins resulted in a greatly enhanced ability of lesioned corticospinal axons to regrow over longer distances.[139] Pesheva and colleagues have studied the JI-160/JI-180 extracellular matrix glyocoproteins, these are of 160 kDa and 180–200 kDa, respectively. They are expressed relatively late in development by oligodendrocytes and by CNS, but not PNS myelin.[140] The JI-160/JI-180 glycoproteins are implicated in an initially adhesive recognition process between oligodendrocytes and neurons that changes into a repulsive one, as a function of time of interaction between these cells.[141] Whilst much of this type of work has, to date, been focused upon the axonal growth inhibitory properties of CNS myelin it should also be noted that Wallerian degeneration is a necessary prerequisite for adult peripheral nerve regeneration. Adult dorsal root ganglion neurons in vitro only extend neurites on predegenerated and not upon normal peripheral nerves.[142] This latter growth response is independent of the presence of NGF and it raises the possibility that PNS myelin may also be inhibitory towards neurite outgrowth from mature neurons.[142]

Conclusions

It seems certain that CNS oligodendrocytes and myelin, possibly also PNS myelin, possess membrane- or extracellular matrix-associated molecules that inhibit the successful regeneration of adult mammalian CNS and PNS axons. The lack of any significant regrowth of lesioned axons in CNS grey matter (where there is minimal myelin) or in myelin-deficient mutant mammals,[143] together with an inability of regenerating dorsal root sensory axons to cross the DRTZ, highlights also the inhibitory role of reactive astrocytes and a relative lack of neurotrophic molecules as being other important factors for CNS regeneration failure. Whilst regenerating CNS axons are strongly inhibited r halted in their regrowth by such influences, it appears that the initially formed growth cones of transplanted foetal CNS neurons, perhaps lacking in or not yet expressing the appropriately responsive receptors, are exempt from such inhibitory molecular influences.

The future

On the basis of what is presently known, it is possible to envisage three directions for the future development of therapeutic approaches towards enhancing neural regeneration after CNS injury. One approach can be summarized as the 'cocktail strategy'. In this, a balanced mixture of neurotrophic factors, together with antibodies to neurite-growth inhibitory molecules present in CNS-myelin and reactive astrocytes, would be administered to regions of CNS damage and/or seeded along the most important neural tracts leading to and from that region. This could be supplemented by PNS conduit grafts to re-establish links between those centres whose interaction are deemed most important. One experimental example of this type of approach has been the guidance, following optic nerve section, of regenerating retinal axons to the pretectal region with successful re-establishment of a pupillary light constrictive reflex.[144] A second approach is, through the use of microtransplanted embryonic donor cell suspensions, to recreate important innervating centres that have been lost or irreparably damaged. This approach was originally developed for the treatment of Parkinsonism and the neurodegenerative disorders.[145–149] More recently, abundant long fibre growth by axons of embryonic hippocampal donor neurons, microtransplanted into the fimbria of immunosupressed hosts has been achieved, the donor axons successfully making contacts with their appropriate terminal fields.[87] Finally, the most exciting and potentially far-reaching therapeutic approach would modify the regenerative growth cones of disconnected but surviving adult central neurons so that

they behave more like the growth cones of foetal neurons growing *ab initio*, i.e. so that they are no longer susceptible to the neurite-growth inhibitory milieu of the adult mammalian CNS. Clearly, however, before this can even begin to be realized, much more will need to be known about the precise nature of the receptors on the membranes of growth cones that respond either to the growth-inhibitory or growth-promoting, molecular influences that exist within the CNS micro-environment.

In looking to the future it is perhaps fitting to end with the words of one whose research at the beginning of this century provided the foundations for so much that has been discovered since.

> The functional specialization of the brain imposed on the neurones two great lacunae; proliferative inability and irreversibility of intraprotoplasmic differentiation. Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated.
>
> It is for the science of the future to change, if possible, this harsh decree. Inspired with high ideals, it must work to impede or moderate the gradual decay of neurones, to overcome the almost invincible rigidity of their connections, and to re-establish normal nerve paths, when disease has severed centres that were intimately associated.
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> Ramon y Cajal, 1914[1]

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